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Acetamiprid-induced testicular toxicity in mice: ameliorative effect and potential mechanisms of morin

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Abstract

Background Acetamiprid (ACP) is a novel chloronicotinyl insecticide that has been extensively utilized in agricultural, domestic, and public health contexts for nearly two decades. However, its potential to induce organ damage, including reproductive toxicity in mammals, has emerged as a significant concern. Morin is a naturally occurring flavonol that has gained prominence as a food supplement in recent years due to its antioxidant and anti-inflammatory properties. The objective of this study was to evaluate the protective effect of morin against testicular damage in mice subjected to ACP exposure.

Methods Thirty male Balb/c mice were randomly assigned to one of five groups, with the following treatment allocations: control, ACP (20 mg/kg), ACP + morin (15 and 30 mg/kg), and only morin (30 mg/kg). ACP and morin applications were conducted orally over a period of 14 days. Hormonal analyses were conducted on serum samples obtained from the mice, while biochemical and histological evaluations were performed on testicular samples.

Results The biochemical results demonstrated that ACP elevated oxidative stress, inflammation, and ER stress in testicular tissue by inhibiting the Nrf2 pathway, a finding that was corroborated by histopathological analyses. However, morin treatments eliminated ACP-induced Nrf2 inhibition and to activate antioxidant and anti-inflammatory mechanisms. These findings were also corroborated by the restoration of serum testosterone and inhibin B levels and the diminution of histopathological lesions.

Conclusions Overall, the findings indicated that morin may have potential protective properties against ACP-associated reproductive toxicity, however, further research is required to determine the detailed molecular mechanisms.

Keywords Acetamiprid, ER stress, Inflammation, Nrf2, Oxidative stress, Testicular toxicity

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Background

Acetamiprid (ACP) is a novel chloronicotinyl insecticide that has gained considerable traction as an alternative to organophosphate and carbamate insecticides [1]. In the field of agriculture, ACP is employed as a means of controlling a range of pests, including leafhoppers, moths, beetles and lepidoptera. Additionally, in the context of domestic and public health, it is utilised as a method of controlling flies, cockroaches, ticks and mites [1, 2]. Although ACP exerts its effects by targeting specific nicotinic acetylcholine receptors (nAChRs) in insects, experimental studies have demonstrated that it also causes toxic effects in mammals [1]. Given that nAChRs are predominantly located in the neuromuscular and reproductive systems of mammals, the tissues most frequently affected by ACP exposure are muscle, nerve and reproductive organs [1, 3, 4]. A number of experimental studies have demonstrated that exposure to ACP is associated with a significant reduction in serum testosterone (TES) levels and sperm count, as well as the degeneration of seminiferous tubules in rodents [1, 2, 5]. The primary mechanism underlying ACP toxicity is the elevation of reactive oxygen species (ROS) levels and the concomitant suppression of the antioxidant system [1]. Experimental evidences have demonstrated that ACP enhances the generation of ROS, predominantly superoxide radical, within tissues. This elevated ROS production results in the damage of antioxidant biomolecules, including superoxide dismutase (SOD), glutathione (GSH) and glutathione peroxidase (GPx), thereby reducing antioxidant capacity and triggering a cascade of reactions that culminate in tissue damage [6, 7]. The elevation of ROS levels following ACP exposure can result in the cumulative damage of tissues. This is occured through the triggering of inflammatory responses, endoplasmic reticulum stress (ERS) and apoptosis mechanisms [8–10]. A substantial body of evidence has demonstrated the pivotal function of nuclear factor erythroid 2-related factor 2 (Nrf2) in initiating antioxidant and cytoprotective processes [11]. The role of Nrf2 in male fertility disorders, including varicocele, cryptorchidism, testicular torsion and orchitis, has been well characterised in research [12]. A body of preclinical research has indicated that Nrf2 levels are diminished in cases of oligospermia [13, 14]. Conclusively, these findings indicate a vital function for Nrf2 in spermatogenesis and the maintenance of sperm quality [12]. The latest research findings underscore the significance of suppressed Nrf2 signalling in ACP toxicity [10, 15]. The pervasive and extensive utilisation of ACP has precipitated a necessity for an appraisal of the toxicological consequences of ACP in mammals and the potential antioxidant and Nrf2 modulator molecules that may mitigate its deleterious effects [1].

The utilisation of natural products in the management of disease has a lengthy historical precedent, spanning centuries [16]. The utilisation of phytochemicals derived from natural products for the prevention and treatment of diseases is becoming increasingly prevalent, due to their favourable toxicity profile and accessibility in comparison to synthetic drugs [11]. Morin (3,5,7,2′,4′-pentahydroxyflavone) is a yellow flavonol that can be isolated from a variety of plant sources, particularly those belonging to the Moraceae family [16]. The distinctive molecular conformation of morin endows it with the capacity to act as a highly efficacious natural radical scavenger [11]. It exhibits a broad spectrum of biological activities, including anti-inflammatory, antioxidant, hepatoprotective, neuroprotective, renoprotective and antidiabetic effects [11, 16]. Recent evidence indicates that morin can confer protection to biological tissues via the modulation of Nrf2 signalling in pathological states typified by oxidative stress (OS) and inflammatory processes [17–19]. Furthermore, it has also been established that the morin can prevent testicular damage induced by acrylamide [20], bisphenol-A [21] and ifosfamide [22]. At present, there is a paucity of evidence to suggest that morin can alleviate testicular damage induced by ACP. The objective of this study was to investigate the hypothesis that morin has the potential to alleviate ACP-induced testicular damage in a mouse model.

Methods

Chemicals and reagents

The ACP (98% purity) provided by Hektaş Ticaret T.A.Ş. (Istanbul, Turkey), was freshly prepared on each study day by dissolving it with 0.5% carboxymethylcellulose (CMC), which had also been used as an inert carrier in previous studies [23, 24]. The morin hydrate was procured from BLD Pharmatech Ltd. (Shanghai, China) and dissolved in a solution comprising 5% dimethyl sulfoxide (DMSO) and 0.5% CMC. This solution was prepared on a daily basis [25]. The enzyme-linked immunosorbent assay (ELISA) kits utilized for the quantification of biochemical parameters were procured from Fine Biotech (Wuhan, China). All other chemicals employed in the study were of analytical grade and procured from Sigma-Aldrich (St. Louis, MO, USA).

Animal housing and management

Thirty adult male Balb/c mice (mean initial weight 25 ± 2 g and four months old) were attained from the Surgical Application Research Center of Karadeniz Technical University. Prior to the commencement of experimentation, the mice were subjected to a one-week acclimation period in metal cages. The mice underwent the experimental procedure within a controlled environment, maintaining a 12-hour light/dark cycle, temperature

of 24 °C and humidity of 60%. In this study, mice were handled humanely to minimise animal suffering in accordance with the ARRIVE guidelines and the UK Animals Act 1986, and all protocols were approved by the Animal Experiments Local Ethics Committee of Karadeniz Technical University (Approval no: 2024/26).

Experimental design

It has been determined that an sample size of six animals per group would provide the appropriate power, according to the formula (1-β = 0.8), to detect significant differences in parameters, given an effect size d = 2.0, a two-sided t-test, and a sample size ratio of 1 (G*Power 3.1.9.2, Kiel University, Kiel, Germany). A total of 30 mice were randomly allocated to one of five treatment groups, and all treatments were administered via intragastrically over the course of 14 days. ***Control group***: Mice received 0.5% CMC intragastrically as a vehicle every day. ***High-dose morin group***: Mice received high dose morin (30 mg/kg) intragastrically every day. ***ACP group***: Mice received ACP (20 mg/kg) intragastrically every day. The dose of ACP employed in the study was determined by considering the relatively mild dose that has been demonstrated to exert toxic effects on the testicle in previous experimental studies [26, 27]. ***ACP + morin group (15 mg/kg)***: The mice were administered ACP (20 mg/kg) intragastrically once a day, and two hours later, morin (15 mg/kg) was administered intragastrically. ***ACP + morin group (30 mg/kg)***: The mice were administered ACP (20 mg/kg) intragastrically once a day, and two hours later, morin (30 mg/kg) was administered intragastrically. The doses of morin used in the study were determined in light of previous reports demonstrating antioxidant and anti-inflammatory activity of morin in experimental models of neuropathic pain [28] and peripheral neuropathy [29]. Twenty-four hours following the administration of the final dose, blood samples were collected via cardiac puncture under general anaesthesia with a mixture of ketamine (60 mg/kg, Vem Pharmaceuticals, Ankara, Türkiye) and xylazine (10 mg/kg, Bayer Global, Leverkusen, Germany) combination. Following blood collection, mice were sacrificed by cervical dislocation for testicle tissue sampling.

Tissue Preparation

The collected testicular tissues were homogenized in phosphate buffered saline (10% w/v), centrifuged at 1800 g for 10 min, and the protein content of the obtained supernatants was determined using the bicinchoninic acid assay [30].

Measurement of lipid peroxidation

The level of malondialdehyde (MDA) was determined as a marker of lipid peroxidation (LPO) in testicular tissues using the colorimetric method [31]. The supernatant was mixed with phosphoric acid and thiobarbituric acid, and then incubated at 90 °C for one hour. Subsequently, the mixture was cooled and then subjected to centrifugation at 1800 g for a period of 10 min. The optical density of the supernatant was determined by a microplate reader (Versamax, Molecular Devices, CA, USA) at 532 nm. The standard employed was 1,1,3,3-tetramethoxypropane, and the MDA level was calculated in nmol/mg protein [32].

Measurement of antioxidant biomarkers

The levels of SOD, GSH, GPx, Nrf2, heme oxygenase-1 (HO-1) and glutamate–cysteine ligase catalytic subunit (GCLC) were determined by ELISA kits, following the methodology outlined by the manufacturer. The following steps were performed in order to execute a typical ELISA measurement. Initially, the standard solution provided by the manufacturer for the relevant parameter was diluted by serial dilution to obtain a series of standard solutions. Subsequently, 100 µL of sample supernatants (in duplicate) and standard solutions were added to the 96-well plate coated with the specific antibody for the parameter to be measured, and the plate was then incubated for 90 min at 37 °C. Subsequent to the completion of this step, the contents of the plate were removed, and the plate was then washed with washing buffer. Thereafter, 100 µL of biotin-labeled antibody was added to each well and the plate was incubated for a further 60 min at 37 °C. Subsequently, the plate content was removed and after washing with washing buffer, 100 µL of horse radish peroxidase solution was added to each well and incubated for 30 min at 37 °C. Subsequently, the plate content was removed and after washing with washing buffer, 90 µL of substrate solution was added to each well and the plate was incubated for 20 min at 37 °C. The reaction was then halted by the addition of 50 µL of stop solution to each well, after which absorbances of all wells were measured at 450 nm using a plate reader (Versamax, Molecular Devices, CA, USA). The concentration-absorbance curve was then plotted for the standards for each parameter, and from the absorbance values of the samples, the levels of the parameter under investigation in the samples were determined in accordance with this curve. The R^2 value of the concentration-absorbance curves generated for all parameters was calculated to be < 0.99.

Measurement of inflammation biomarkers

The levels of high mobility group box 1 (HMGB1), nuclear factor kappa-B (NF-κB) p65, interleukin-6 (IL-6) and myeloperoxidase (MPO) were determined by ELISA kits, following the methodology outlined by the manufacturer.

Measurement of ERS and apoptosis biomarkers

The levels of heat shock protein family A member 5 (HSPA5), activating transcription factor 6 (ATF6), DNA damage-inducible transcript 3 (DDIT3) and cleaved caspase-3 (CASP3) were determined by ELISA kits, following the methodology outlined by the manufacturer.

Measurement of serum testosterone and inhibin B levels

The serum concentrations of TES and inhibin B (INHB) were determined by ELISA kits, following the methodology outlined by the manufacturer.

Histopathological analysis

For histological examination, the right testicular samples were fixed in Bouin's solution for a period of two days, subsequently dehydrated using a graded alcohol series, and finally embedded in paraffin. Four-micrometre sections were excised from the paraffin blocks using a semi-automatic microtome and subjected to staining with haematoxylin and eosin (H&E) [27, 33]. The stained slides were examined and photographed by a histologist who was blinded to the groups under a light microscope combined with a digital camera. The images displayed on the slides were captured at 200x and 400x magnifications. The images were evaluated according to the following criteria and scored between 0 and 3 according to the frequency of pathological findings (0: none, 1: mild, 2: moderate and 3: severe): the presence of germinal epithelial cells in the lumen, the accumulation of seminiferous tubule epithelium towards the lumen, vasocongestion and irregularities among seminiferous tubule epithelial cells [34].

Statistical analysis

All results were expressed as mean ± standard error of the mean (SEM). The conformity of the data to a normal distribution was evaluated using the Shapiro-Wilk test. The data for multiple variable comparisons were subjected to analysis using ANOVA, followed by a Tukey post-hoc test, with the software SPSS (23.0). The level of significance deemed acceptable was set at $p < 0.05$.

Results

No deaths were observed in any of the experimental groups during the course of the experiment. Furthermore, no clinical indications of ACP poisoning were evident in the treated mice.

Impacts of morin on antioxidant/oxidant markers in ACP-treated mice

The administration of oral ACP was found to significantly elevate the levels of LPO in comparison to the control group ($p = 0.0001$), while concurrently reducing the activity of the antioxidant system, including GSH (~3.2 fold; $p = 0.008$), SOD (~3.3 fold; $p = 0.028$) and GPx (~3.5 fold; $p = 0.034$). In comparison to the ACP group, low-dose morin supplementation yielded a notable restoration of only MDA levels ($p = 0.0001$). Nevertheless, in comparison to the ACP group, high-dose morin supplementation significantly enhanced the antioxidant system and reduced LPO levels (Table 1).

The levels of Nrf2 (~3.0 fold; $p = 0.026$), HO-1 (~5.2 fold; $p = 0.026$) and GCLC (~3.4 fold; $p = 0.007$) were found to be significantly reduced in the testicular tissue of mice that had been exposed to ACP, in comparison to control mice. Nevertheless, the levels of Nrf2 ($p = 0.016$), HO-1 ($p = 0.028$), and GCLC ($p = 0.017$) were markedly elevated in the high dose morin-supplemented group in comparison to the ACP group (Fig. 1). However, there was no statistically significant difference between morin treatment groups in terms of lipid peroxidation and antioxidant system parameters ($p > 0.05$).

Impacts of morin on inflammation markers in ACP-treated mice

In comparison to the control group, ACP exposure resulted in the induction of inflammation in the testicular tissue of mice, as evidenced by elevated levels of HMGB-1 (~3.1-fold; $p = 0.004$), NF-κB (~7.6-fold; $p = 0.0001$), IL-6 (~2.7-fold; $p = 0.0001$) and MPO (~10.0-fold; $p = 0.001$). Nevertheless, both doses of morin supplementation demonstrated a notable improvement in testicular inflammation resulting from ACP exposure, when compared to the ACP group (Fig. 2). However, there was no statistically

Table 1 Effect of administration of morin on markers of OS in testicular tissue

	Control	Morin (30 mg/kg)	ACP	ACP + Morin (15 mg/kg)	ACP + Morin (30 mg/kg)
MDA (nmol/mg protein)	3.05 ± 0.70	2.80 ± 0.32	20.01 ± 4.03***	4.17 ± 0.60$^{\#\#\#}$	2.69 ± 0.37$^{\#\#\#}$
GSH (µg/mg protein)	16.62 ± 2.72	17.06 ± 1.64	5.21 ± 1.58**	10.35 ± 1.18	18.90 ± 3.08$^{\#\#}$
SOD (ng/mg protein)	0.91 ± 0.36	1.00 ± 0.29	0.28 ± 0.02*	0.44 ± 0.06	0.77 ± 0.03$^{\#}$
GPx (IU/mg protein)	4.58 ± 0.24	5.43 ± 1.08	1.31 ± 0.42*	3.61 ± 0.58	4.58 ± 1.00$^{\#}$

ACP: acetamiprid, MDA: malondialdehyde, GSH: glutathione, SOD: superoxide dismutase, GPx: glutathione peroxidase. Data are represented as mean ± SEM ($n = 6$)

Different groups were analyzed using one-way ANOVA following by Tukey's multiple comparison test

$^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared with control group

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ when compared with only ACP treated group

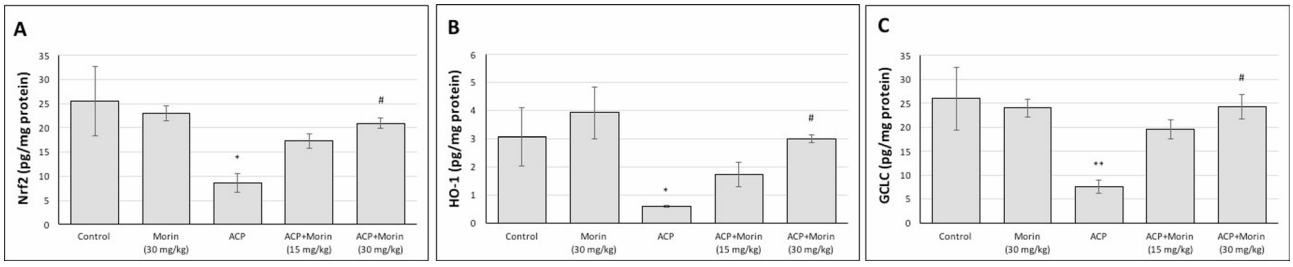

Fig. 1 Effect of morin on the levels of Nrf2 (**A**), HO-1 (**B**) and GCLC (**C**) in the testicles of mice. Data are represented as mean ± SEM ($n = 6$). Different groups were analyzed using one-way ANOVA following by Tukey's multiple comparison test. $^{*}p < 0.05$ and $^{**}p < 0.01$ when compared with control group. $^{\#}p < 0.05$ when compared with only ACP treated group

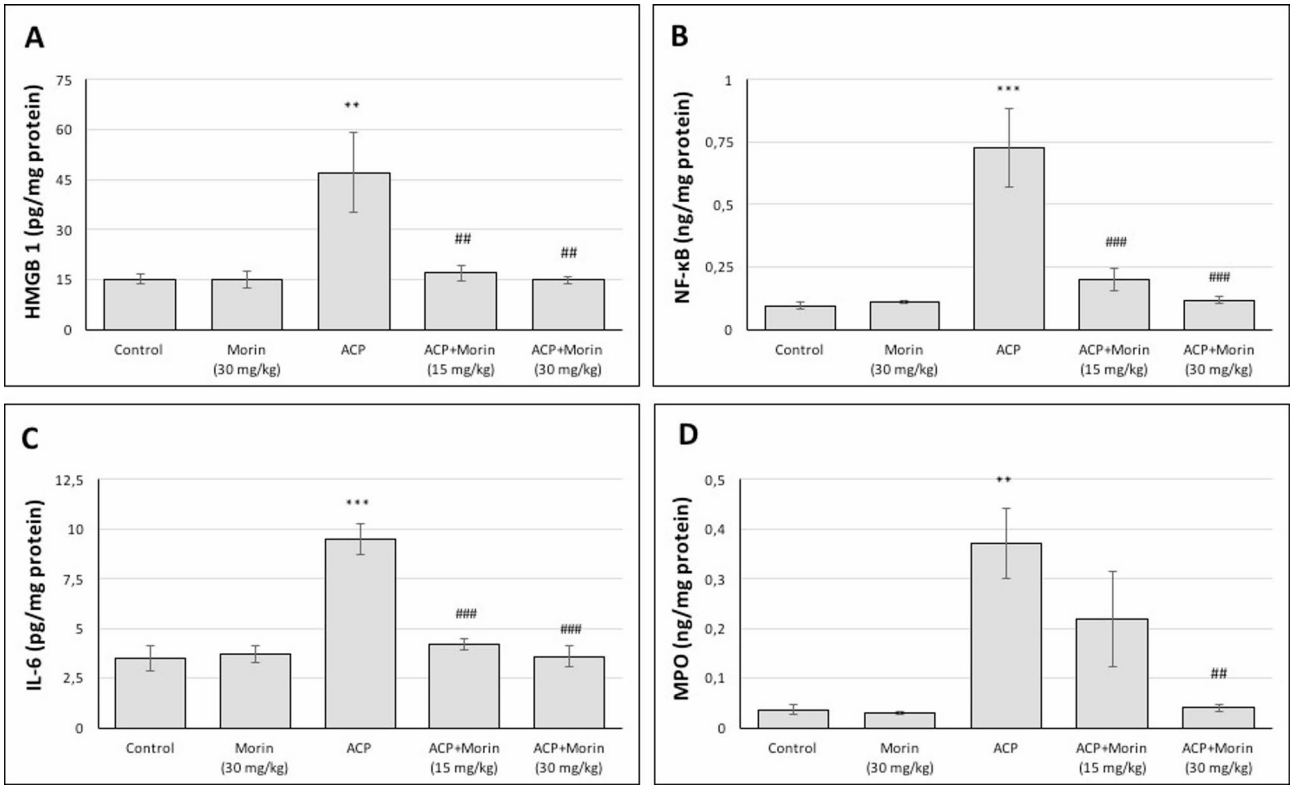

Fig. 2 Effect of morin on the levels of HMGB1 (**A**), NF-κB (**B**), IL-6 (**C**) and MPO (**D**) in the testicles of mice. Data are represented as mean ± SEM ($n = 6$). Different groups were analyzed using one-way ANOVA following by Tukey's multiple comparison test. $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared with control group. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ when compared with only ACP treated group

significant difference between morin treatment groups in terms of inflammation parameters ($p > 0.05$).

Impacts of morin on ERS and apoptosis markers in ACP-treated mice

The administration of ACP elevated the levels of ERS and apoptosis markers in testicular tissue, as evidenced by the elevated expression of HSPA5 ($p = 0.0001$), ATF6 ($p = 0.0001$), DDIT3 ($p = 0.004$) and CASP3 ($p = 0.0001$) when compared to the control group. Nevertheless, both doses of morin supplementation demonstrated a notable improvement in testicular ERS and apoptosis resulting from ACP exposure, when compared to the ACP group (Fig. 3). However, there was no statistically significant difference between morin treatment groups in terms of ERS and apoptosis parameters ($p > 0.05$).

Impacts of morin on male reproductive hormones in ACP-treated mice

The administration of oral ACP resulted in a notable reduction in serum TES ($p = 0.0001$) and INHB ($p = 0.0001$) concentrations when compared to the control group. Conversely, the administration of morin supplements at both doses resulted in a significant elevation in serum TES and INHB levels when compared to the ACP group In addition, INHB levels were found to be

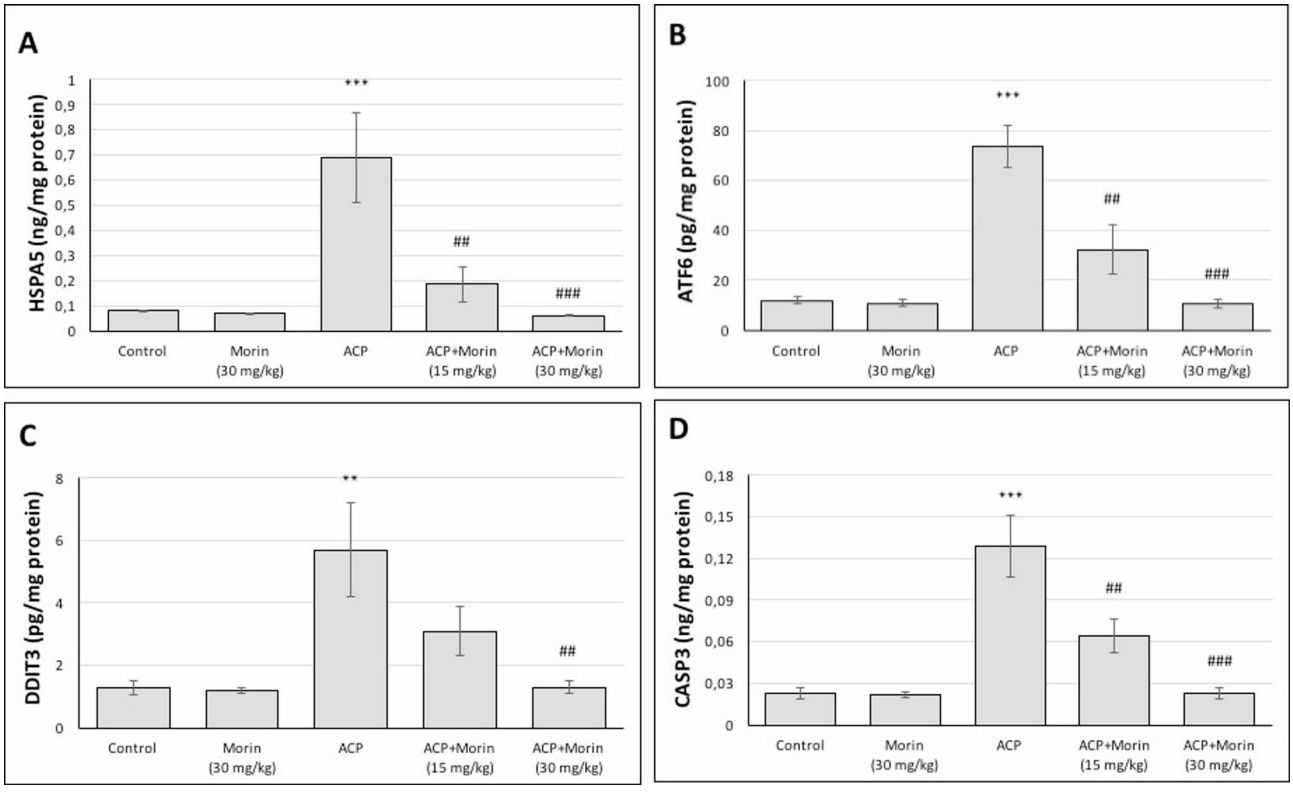

Fig. 3 Effect of morin on the levels of HSPA5 (**A**), ATF6 (**B**), DDIT3 (**C**) and CASP3 (**D**) in the testicles of mice. Data are represented as mean ± SEM ($n = 6$). Different groups were analyzed using one-way ANOVA following by Tukey's multiple comparison test. $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared with control group. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ when compared with only ACP treated group

Table 2 Effect of administration of morin on serum TES and INHB levels

	Control	Morin (30 mg/kg)	ACP	ACP + Morin (15 mg/kg)	ACP + Morin (30 mg/kg)
TES (ng/mL)	14.83 ± 1.19	18.21 ± 1.09	$3.10 \pm 0.48^{***}$	$8.86 \pm 1.47^{*,\#}$	$13.74 \pm 1.98^{\#\#\#}$
INHB (pg/mL)	1580.3 ± 8.7	1465.7 ± 36.5	$389.9 \pm 86.3^{***}$	$821.2 \pm 23.0^{***,\#\#\#}$	$1476.6 \pm 48.2^{\#\#\#,+++}$

ACP: acetamiprid, TES: testosterone, INHB: inhibin B. Data are represented as mean ± SEM ($n = 6$)

Different groups were analyzed using one-way ANOVA following by Tukey's multiple comparison test

$^{*}p < 0.05$ and $^{***}p < 0.001$ when compared with control group

$^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ when compared with only ACP treated group

$^{+++}p < 0.001$ when compared with ACP + morin (15 mg/kg) group

statistically significantly higher in the high-dose morin treatment group than in the low-dose morin treatment group ($p = 0.0001$) (Table 2).

Impacts of morin on histological lesions in ACP-treated mice

The Fig. 4 illustrated the histological changes that were observed in the testicular specimens from each of the experimental groups, and Table 3 represented the semi-quantitative scores of these histopathological findings. The testicular tissue from the control group exhibited normal seminiferous tubule and interstitial area architecture. While the seminiferous tubules and interstitial areas of the testicular tissue in the high-dose morin (30 mg/kg) group exhibited normal testicular architecture, some regions displayed evidence of vasocongestion between the seminiferous tubules. In the ACP group, the seminiferous tubule epithelium was observed to exhibit a piled-up configuration towards the lumen, as well as openings and irregularities between epithelial cells and germinal epithelial cells within the lumen. Furthermore, extensive vasocongestion was evident in the intertubular regions (as indicated by higher scores). In the ACP + morin (15 mg/kg) group, although there was a slight decrease in the number of seminiferous tubule epithelial cells observed compared to the ACP group, there was a notable accumulation of these cells towards the lumen, as well as the presence of irregularities between the epithelial cells. Furthermore, vasocongestion was observed in the intertubular areas. However, the improvements observed

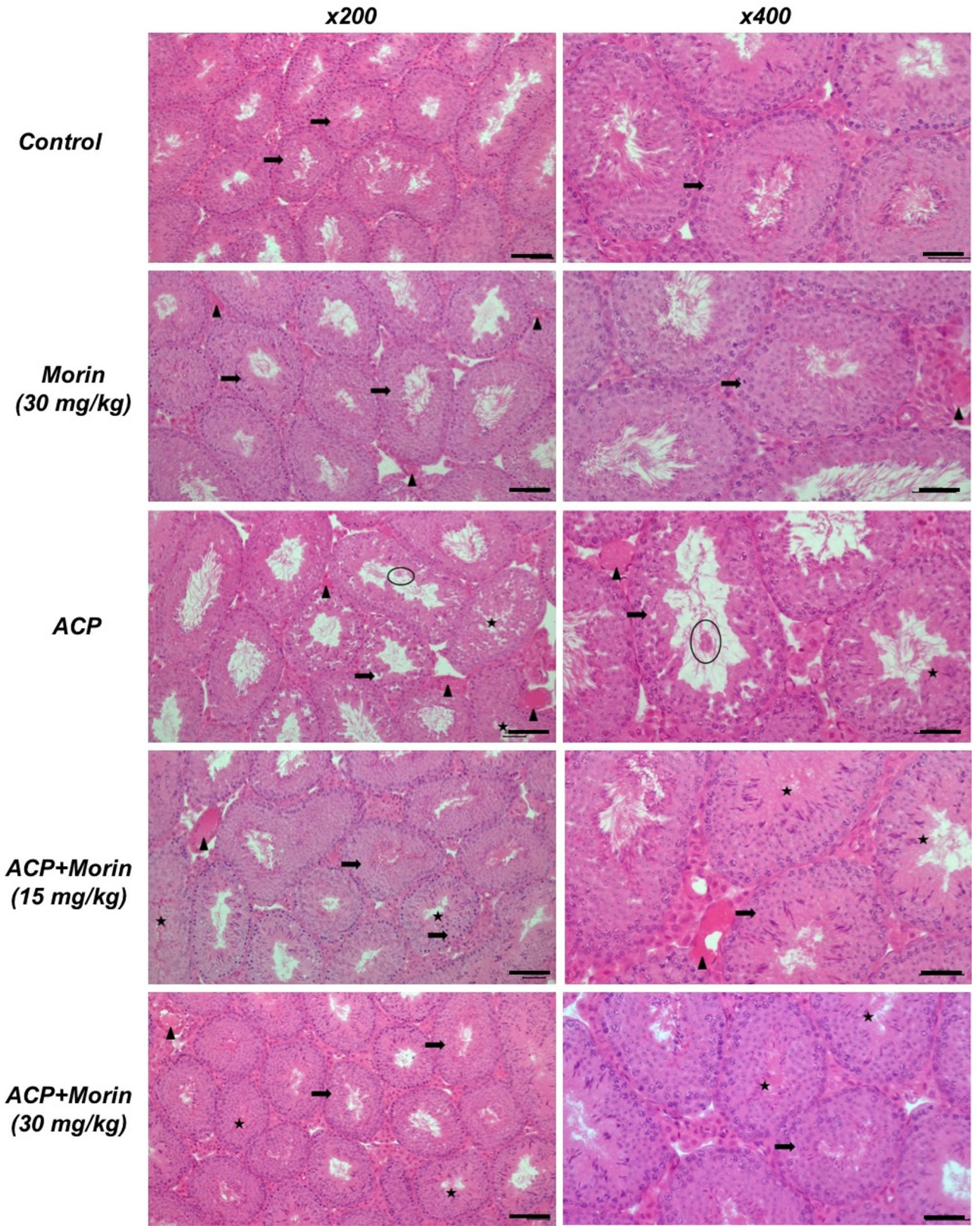

Fig. 4 (See legend on next page.)

(See figure on previous page.)

Fig. 4 The light microscopy images of H&E-stained testicles from the control and experimental groups. The images have been captured at two different magnifications: ×200 and ×400, respectively. In the slides of control group, the architecture of the normal seminiferous tubule (black arrow) and interstitial space was predominant. The examination of the slides from the only morin treatment group revealed the presence of normal seminiferous tubule (black arrow) and interstitial space morphology. However, mild vasocongestion (arrowhead) was observed between the seminiferous tubules. In the slides from the ACP group, moderate the accumulation of seminiferous tubule epithelium towards the lumen (star) with moderate irregularities between the epithelial cells, disrupted seminiferous tubule structures (black arrow) and mild germinal epithelial cells in the lumen (circle) were obtained. Furthermore, moderate vasocongestion (arrowhead) findings were identified in the intertubular areas. In the slides taken from the ACP + morin (15 mg/kg) group, it was determined that the accumulation of seminiferous tubule epithelium towards the lumen was moderate (star). Furthermore, although these findings were found to be lower in comparison to the ACP group, mild irregularities disrupted seminiferous tubule structures (black arrow) and vasocongestion (arrowhead) findings were identified. In the slides taken from the ACP + morin (30 mg/kg) group, although the testicular seminiferous tubule architecture (black arrow) was close to normal, mild accumulation of seminiferous tubule epithelium towards the lumen (star) and vasocongestion (arrowhead) findings were observed

in the ACP + morin (15 mg/kg) group compared with the ACP-only group were not statistically significant on the semi-quantitative scores ($p > 0.05$). The testicular seminiferous tubule and interstitial area morphology in the ACP + morin (30 mg/kg) group was observed to be similar to that of a normal control group. Compared with the ACP + morin (15 mg/kg) group, there was a significant decrease in the number of cases where seminiferous tubule epithelial cells accumulated towards the lumen. However, this decrease was not statistically significant ($p > 0.05$). Although a very uncommon occurrence, instances of vasocongestion were observed in the intertubular areas.

Discussion

The objective of this study was to ascertain whether morin can provide protection for the testes of mice that had been treated with ACP for the first time. In line with the preceding evidence derived from experimental models [2, 24, 35], our findings revealed that only the mice administered with ACP exhibited notable degenerative alterations in their testes, accompanied by a significant reduction in serum TES and INHB levels. TES is a vital hormone for the process of spermatogenesis [36]. INHB is a hormone produced by Sertoli cells that exerts control over the process of spermatogenesis by providing feedback inhibition of FSH release [37]. TES and INHB hormones play a pivotal role in spermatogenesis, and maintaining equilibrium between these hormones is essential for optimal testicular function [24]. The cyclic adenosine monophosphate (cAMP) is a molecule that plays a role in TES biosynthesis by regulating the enzymes involved in the process of steroidogenesis, including 3 beta-hydroxysteroid dehydrogenase. However, the increase in ROS caused by ACP has a detrimental impact on cAMP levels [1, 2]. We obtained findings reflecting increased OS in the ACP, as demonstrated by higher LPO levels (as revealed by higher MDA levels) and decreased antioxidant capacity (as revealed by lower SOD, GSH and GPx levels). It can therefore be surmised that the marked decline in TES levels observed in the ACP group may be attributable to the depletion of the proteins that regulate TES synthesis from cholesterol in the testicular tissue of mice. Nevertheless, our findings indicated that morin treatments resulted in elevated serum TES and INHB levels in ACP-treated mice. This was corroborated by the diminution of histopathological lesions in comparison to the ACP group. When considered together with our data, which indicate that morin treatments reduced ACP-induced OS in testicular tissue (lower LPO levels and higher antioxidant capacity), morin administration may help to maintain the physiological functions of Leydig and Sertoli cells by reducing the amount of ROS. Our findings align with the results of prior research, indicating that morin exerts testicular protective effects through its antioxidant capacity [22, 36, 38].

The most crucial factor in the activation of defensive mechanisms against OS is Nrf2 [11]. The expression of numerous antioxidant enzymes, including GCLC (involved in glutathione synthesis) and HO-1, is initiated by the dissociation of cytoplasmic Nrf2 from Kelch-like ECH-associated protein1 and subsequent migration to the nucleus [22]. The application of various pesticides, including ACP, has been demonstrated to induce OS by suppressing Nrf2 level [10, 15]. The current study corroborates the findings of above previous research, demonstrating that ACP reduce HO-1 and GCLC levels by inhibiting the Nrf2 pathway in testicular tissue. Conversely, morin treatments were observed to eliminate the Nrf2 inhibition induced by ACP. These findings were in alignment with the conclusions of previous studies that have demonstrated morin's ability to modulate the Nrf2 pathway in a variety of experimental models [17–19]. Despite the fact that the mechanism through which phenolic compounds offer protection to tissues against OS has been investigated principally in the context of quenching LPO directly and inducing the antioxidant system, recently gathered data indicates that phenolic compounds may also provide protection in indirect way by regulating the expression of genes that offer protection to cells via Nrf2 [39]. The Nrf2 modulatory activity of phenolic compounds is likely due to their low concentrations, which act as a catalyst for Nrf2, thereby exerting

Table 3 The degree of histopathological lesions in testicular tissues of different treatment groups

	Control	Morin (30 mg/kg)	ACP	ACP + Morin (15 mg/kg)	ACP + Morin (30 mg/kg)
The presence of germinal epithelial cells in the lumen	0.33 ± 0.21	0.33 ± 0.21	1.33 ± 0.21*	0.67 ± 0.21	0.50 ± 0.22$^{\#}$
The accumulation of seminiferous tubule epithelium towards the lumen	0.33 ± 0.21	0.50 ± 0.22	2.33 ± 0.21***	1.50 ± 0.22**	0.67 ± 0.21$^{\#\#\#}$
Vasocongestion	0.33 ± 0.21	0.50 ± 0.22	1.67 ± 0.21**	1.17 ± 0.17	0.83 ± 0.31$^{\#}$
Irregularities among seminiferous tubule epithelial cells	0.33 ± 0.21	0.67 ± 0.21	2.17 ± 0.31***	1.33 ± 0.21	0.67 ± 0.33$^{\#\#}$

ACP: acetamiprid. Data are represented as mean ± SEM ($n = 6$)

Different groups were analyzed using one-way ANOVA following by Tukey's multiple comparison test

$^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ when compared with control group

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ when compared with only ACP treated group

potent effects on pro-oxidative and inflammatory mediators [40]. It has been well defined that not only morin but also other flavonols, such as kaempferol, myricetin and quercetin, have Nrf2 modulatory properties [39, 40].

Inflammation is a biological response that promotes survival when cells are exposed to a chemical, physical, or biological agent [20]. However, chronic inflammation causes permanent cell and tissue damage [41]. HMGB1 is a non-histone chromatin-associated protein that binds to toll-like receptor 4, thereby activating NF-κB pathway and resulting in the elevation of inflammatory cytokines, including IL-6 [42]. It can therefore be concluded that the inhibition of the HMGB1/ NF-κB/IL-6 axis represents a potential therapeutic target for the treatment of pathologies characterised by chronic inflammation [32, 43]. In similar to the findings of previous experimental studies [9, 10, 44], our research demonstrated that ACP administration resulted in elevated levels of inflammation in testicular tissue. In contrast, morin treatments significantly attenuated testicular inflammation by inhibiting the HMGB1/NF-κB axis. It is established that Nrf2 exerts a negative regulatory effect on the HMGB1/NF-κB axis [45, 46]. The observation of elevated inflammatory markers in the ACP group, despite lower Nrf2 levels, lends support to this hypothesis. Nevertheless, the reduction of inflammatory levels with morin treatment can be attributed to both the previously demonstrated in vivo anti-inflammatory activity of morin and the inhibition of the HMGB1/NF-κB axis by restoration of suppressed Nrf2 levels, as previously demonstrated [11, 16].

A reduction in the ability of the ER to fold proteins can result from a number of conditions, including redox imbalance, calcium imbalance, hypoxia and nutrient depletion. The accumulation of unfolded proteins that results from this is known as ERS [47]. Disturbance in ER homeostasis is detected by three sensor proteins (including ATF6), activating the signaling pathway called the unfolded protein response (UPR) [48]. In homeostatic circumstances, ATF6 is in direct physical contact with HSPA5 and remains in an inactive state [49]. However, following the activation of UPR, ATF6 is released and subsequently activated within the Golgi apparatus. This leads to its migration to the nucleus, where it induces the transcription of a multitude of genes, including DDIT3 [48]. While UPR activation is instrumental in maintaining tissue survival in acute conditions, DDIT3-mediated apoptosis is promoted in chronic ERS conditions [47]. A number of studies have demonstrated that ERS and ERS-related apoptosis contribute to the damage observed in cells following ACP-induced toxicity [1, 8]. The results of our study corroborated those of above previous research, indicating that ACP administration results in cell death by increasing ERS and apoptosis levels. Conversely, the co-administration of morin and ACP was observed to reverse the ERS and apoptosis levels in comparison to mice exposed to ACP alone, thereby improving testicular tissue. It is evident that Nrf2 plays a pivotal role in alleviating ERS. This is achieved through two principal mechanisms: firstly, by stimulating the antioxidant response and secondly, by up-regulating the expression of proteasome catalytic subunits and enhancing proteasome-mediated ER-associated degradation [47]. The observation that lower Nrf2 levels in the ACP group were accompanied by higher ERS and apoptosis levels lends support to this hypothesis. Nevertheless, the restoration of Nrf2 levels in the morin treatment group in comparison to the ACP group may have contributed to the alleviation of ERS. Our findings were also consistent with those of previous studies which have demonstrated that morin can alleviate ERS [22, 50, 51].

The present study was subject to a number of limitations. The present study was subject to a number of limitations. Firstly, the study evaluated the testicular protective effect of two doses of morin (15 and 30 mg/kg), which are frequently preferred in the literature, against 14-day ACP exposure. The extent to which this protective effect of morin will be effective if the ACP exposure period is prolonged remains to be elucidated. Secondly, the evaluation of biochemical parameters by ELISA method was necessitated by financial constraints. In future studies, the determination of biochemical parameters related to cell signalling by western blotting, immunohistochemistry and/or RT-qPCR techniques will facilitate a more detailed elucidation of the molecular

mechanism. Thirdly, the addition of positive and/or negative control treatment groups to future studies may prove beneficial in determining the role of ERS in ACP-induced testicular damage with greater comprehensiveness. Finally, the effects of ACP exposure and morin treatments on sperm parameters and physiological fertility performance of male mice could not be determined. It is recommended that these parameters be incorporated into subsequent studies to enhance the comprehensiveness and robustness of the research.

Conclusion

The results of our study demonstrated that ACP-induced testicular dysfunction can be alleviated by morin treatment, with a greater efficacy observed at higher dose (30 mg/kg). It is hypothesised that the improvement in testicular function observed following morin treatment may be attributed to the reduction of oxidative stress, inflammation, ERS and apoptosis via Nrf2 modulation. While these findings suggest that morin may serve as a promising protective agent against testicular toxicity induced by ACP exposure, further comprehensive molecular studies are necessary before its clinical application.

Abbreviations

ACP	Acetamiprid
ATF6	Activating transcription factor 6
cAMP	Cyclic adenosine monophosphate
CASP3	Cleaved caspase-3
CMC	Carboxymethylcellulose
DDIT3	DNA damage-inducible transcript 3
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
ERS	Endoplasmic reticulum stress
GCLC	Glutamate-cysteine ligase catalytic subunit
GPx	Glutathione peroxidase
GSH	Glutathione
H&E	Haematoxylin and eosin
HMGB1	High mobility group box 1
HO-1	Heme oxygenase-1
HSPA5	Heat shock protein family A member 5
IL-6	Interleukin-6
INHB	Inhibin B
LPO	Lipid peroxidation
MDA	Malondialdehyde
MPO	Myeloperoxidase
nAChRs	Nicotinic acetylcholine receptors
NF-κB	Nuclear factor kappa-B
Nrf2	Nuclear factor erythroid 2-related factor 2
OS	Oxidative stress
ROS	Reactive oxygen species
SEM	Standard error of the mean
SOD	Superoxide dismutase
TES	Testosterone
UPR	Unfolded protein response

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Author contributions

NTA and SD conceived the study and coordinated all laboratory experiments. NTA, SD and EAD performed the biochemical analysis. EY, AK and NSE performed the histological analysis. EY and YA contributed to methodology. NTA, SD, EY and AM performed the data analysis. NTA and SD wrote the manuscript. EY and YA amended the manuscript. All authors read and approved the manuscript.

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Data availability

All data obtained from this study are included in the current manuscript.

Declarations

Ethics approval and consent to participate

This study was approved by the Local Animal Research Ethics Committee of Karadeniz Technical University (Protocol no: 2024/26) and conducted in accordance with the animal research reporting of in vivo experiments (ARRIVE) guidelines and the UK Animals (Scientific Procedures) Act 1986 and related guidelines, EU Directive 2010/63/EU for animal experimentation and National Institutes of Health guidelines.

Competing interests

The authors declare no competing interests.

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